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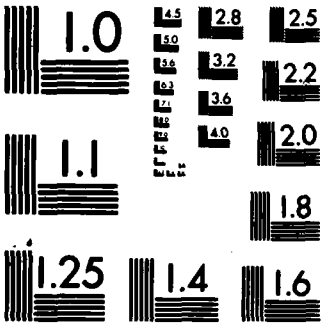

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MONKEY RED BLOOD CELLS FROZEN WITH 40% (w/v) GLYCEROL AND STORAGE AT -80 C

by

C. R. VALERI, D. A. VALERI, A. GRAY, P. D. LEAVY,

T. J. CONTRERAS, AND J. R. LINDBERG

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

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Red blood cells of Rhesus monkeys cryopreserved with 40% (w/v) glycerol and storage at -80 C had freeze-thaw-wash recovery values of 87%, 24-hour post-transfusion survival values of 85%, and lifespan values of 13 days. Our data show that the monkey is an excellent model in which to study liquid and freezing methods of preserving RBC before conducting tests in humans.

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SUMMARY

Red blood cells of Rhesus monkeys cryopreserved with 40% (w/v) glycerol and storage at -80 C had freeze-thaw-wash recovery values of 87%, 24-hour posttransfusion survival values of 85%, and lifespan values of 13 days. Our data show that the monkey is an excellent model in which to study liquid and freezing methods of preserving RBC before conducting tests in humans.

INTRODUCTION

Monkeys, as well as chimpanzees, gibbons, and baboons, have been used to study methods of preserving RBC in the liquid and frozen states,[1-6] and the RBC of monkeys have been shown to act similarly to human RBC when treated with a solution of inosine, pyruvate and inorganic phosphorus to increase red cell 2,3 DPG levels.[7] The monkey also has been shown to be useful as an experimental model for the evaluation of pharmacologic methods used in the in vivo alteration of the RBC oxygen-hemoglobin equilibrium curve.[8]

This paper reports the successful freeze-preservation of the RBC of Rhesus monkeys using 40% (w/v) glycerol and frozen storage at -80 C. The recovery of RBC after the freeze-thaw-wash procedure and the survival of fresh and cryopreserved monkey RBC are reported.

MATERIALS AND METHODS

Five male Rhesus monkeys (Macacca Mulatta) weighing about 12 kg were bled 100 ml volumes of blood; some of the units were studied as fresh RBC and some as cryopreserved RBC. The blood was collected under sterile conditions into 14 ml of citrate-phosphate-dextrose (CPD) anticoagulant in a 300 ml polyvinylchloride (PVC) plastic bag and was stored at 4 C for 4 to 6 hours.

Cryopreserved RBC were prepared as follows: The blood was placed in a plastic overwrap and incubated in a water bath maintained at 37 C for 30 minutes to warm to a temperature of 22 C to 30 C. Following this period of incubation, the blood was centrifuged at 4200 rpm (4500 X g) for 5 minutes in a refrigerated centrifuge[a] maintained at 22 C, and all the plasma was expressed into a transfer pack. Fifteen ml of 0.9% sodium chloride solution was added to eliminate spontaneous agglutination of the RBC.

The RBC were glycerolized to a final concentration of 40% (w/v) and frozen and stored at -80 C. A 6.2 M glycerol solution containing per 100 ml: 57.1 g glycerol, 0.03 g potassium chloride, 0.04 g magnesium chloride, 1.6 g sodium lactate, and 0.08 g disodium phosphate, adjusted to a pH of 6.8, was added to the RBC as follows: Using a modified Eberbach shaker, a weight of the glycerol solution equal to the weight of the RBC-sodium chloride mixture was added in 5 steps. In each of the first two additions, 10% of the total glycerol solution was added over a 5-minute

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[a]RC-3, DuPont Instruments, Newtown, CT

period with mixing, followed by a 10-minute period of equilibration at room temperature without mixing. In the third and the fourth additions, 25% of the total glycerol was added over a 5-minute period with mixing, followed by 5 minutes of equilibration at room temperature without mixing. In the fifth addition, the last 30% of the glycerol was added over a 5-minute period with agitation, followed by a 10-minute period of equilibration without agitation.

The glycerolized RBC were transferred to a polyolefin plastic bag, placed in an aluminum container, and frozen in a -80 C mechanical freezer. The frozen RBC were stored at -80 C for up to 42 days.

Thawing and Washing--The frozen RBC were thawed with mechanical agitation in a 37 C water bath in about 5 minutes. Then a 12% sodium chloride solution equivalent to 25% of the weight of the RBC-glycerol mixture was added with manual agitation in about 2 minutes, followed by equilibration at room temperature for 10 minutes without mixing. Next, a volume of 1.6% sodium chloride solution equivalent to 200% the weight of the RBC-glycerol mixture was added slowly in 4 equal parts, each addition taking about 2 minutes and each followed by 5 minutes of equilibration at room temperature without mixing.

Upon completion of the dilution process, the RBC were recovered in the washing bag of the IBM Blood Processor 2991-1 or 2991-2.[b] The recovered RBC were concentrated by centrifugation at 3000 rpm for 2.5 minutes, and the supernatant solution was decanted at a rate of 450 ml/min. The rate of pump restoration was set at 300 ml/min, and the supernatant volume was

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[b]IBM Corp., Princeton, NJ

adjusted to 600 ml.

A 250 ml volume of 1.6% sodium chloride was added to the RBC with mixing. Following concentration of the RBC at 3000 rpm for 1.5 minutes and decantation of the supernatant solution, another 250 ml volume of 1.6% sodium chloride was added to the RBC as described above. The RBC were diluted twice, each time with a 250 ml volume of sodium chloride (0.9 g/dl)-glucose (0.2 g/dl)-Na_2HPO_4 (0.065 g/dl) added with agitation at 100 ml/min and each followed by centrifugation to remove the supernatant solution. The PCV was adjusted to approximately 40% by the addition of 50 ml of NaCl-glucose-phosphate solution. The washed RBC were transferred to a 300 ml transfer pack and stored at 4 C for 24 hours prior to autotransfusion.

In Vitro Measurements--In vitro recovery values were measured after the freeze-thaw and freeze-thaw-wash procedures, and extracellular potassium and supernatant hemoglobin levels were measured as previously described.[9,10] Both on the day of blood collection and on the day of RBC postthaw washing, measurements were made of RBC mean corpuscular volume (MCV, u^3), mean corpuscular hemoglobin (MCH, uug), mean corpuscular hemoglobin concentration (MCHC, %), RBC sodium, potassium, 2,3 DPG, ATP, and P_{50} values, as previously described.[9,10]

In 3 monkeys autotransfused with fresh ^{51}Cr-labeled red blood cells, plasma volume was measured using ^{125}I-labeled albumin, and total body hematocrit was calculated.[11] The blood for these studies was collected in acid-citrate-dextrose (ACD, NIH, Formula A), labeled with sodium ^{51}Cr chromate, and reinfused to the monkeys within 4 hours of collection. The

factor (f factor) relating the peripheral venous hematocrit to the total body hematocrit was derived.[12] Washed previously frozen RBC also were autotransfused, and 24-hour posttransfusion survival was measured by the ^{51}Cr labeling technique. The RBC volume was estimated from the plasma volume measured using ^{125}I-labeled albumin, and the total body hematocrit was calculated from the peripheral venous hematocrit multiplied by the "f" factor.[5,6] The T_{50} values for both the fresh and the washed previously frozen RBC were determined from the time required for the removal of 50% of the RBC-associated radioactivity after transfusion. The index of therapeutic effectiveness of cryopreserved RBC was calculated from the in vitro recovery of RBC after the freeze-thaw-wash procedure multiplied by the 24-hour posttransfusion survival value.[13]

RESULTS

When fresh RBC were autotransfused to 3 monkeys, the "f" factor, which relates the peripheral venous hematocrit to the total body hematocrit, was 0.90; the T_{50} was 13.5 days (Table 1 and Figure 1). TABLE 1 FIG. 1

Table 2 reports the 2,3 DPG, ATP, P_{50}, potassium and sodium levels in fresh RBC from 4 monkeys. TABLE 2

Five units of monkey RBC frozen with 40% (w/v) glycerol and stored at -80 C for up to 42 days had freeze-thaw recovery values of 98.7% and freeze-thaw-wash recovery values of 87.4% (Table 3). The mean 24-hour posttransfusion survival value in three of these units after 24 hours of post-wash storage at 4 C was 84.7% (Table 3). TABLE 3

Three units of freeze-preserved monkey RBC had T_{50} values of 13 days (Figure 2). Table 3 also reports levels of RBC 2,3 DPG, ATP, potassium and sodium, supernatant hemoglobin, and extracellular potassium on the day of washing. FIG. 2

DISCUSSION

Monkey RBC have been satisfactorily frozen with 40% (w/v) glycerol and storage at -80 C. The mean freeze-thaw-wash recovery value was 87%, the mean 24-hour posttransfusion survival value was 85%, and the lifespan value was 13 days.

Monkeys can be used to study new methods of liquid and cryopreservation of RBC so that the therapeutic effectiveness and safety of the procedures can be verified before human volunteers are subjected to in vivo studies.

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TABLE 1--Red Blood Cell Volume, Plasma Volume, Total Blood Volume, and the "f" Factor in the Rhesus Monkey

Monkey No.	Weight (kg)	^{51}Cr RBC Volume (ml)	^{125}I Albumin Plasma Volume (ml)	Total Blood Volume (ml)	"f" Factor
1	11.5	257	341	598	0.91
2	12.9	224	364	588	0.89
3	11.8	211	313	524	0.91
Mean	12.1	231	339	570	0.90
SD	0.7	24	26	40	0.01
SE	0.4	14	15	23	0.01
n	3	3	3	3	3

TABLE 2--In Vitro Measurements of Fresh Rhesus Monkey Blood

	RBC 2,3 DPG (uM/g Hb)	RBC ATP (uM/g Hb)	RBC K^+ (mEq/10^{12} RBC)	RBC Na^+ (mEq/10^{12} RBC)	RBC P_{50} (mm Hg)
Mean	16.0	3.1	7.29	1.27	33.4
SD	0.5	0.6	0.36	0.08	0.7
SE	0.2	0.3	0.18	0.03	0.4
n	4	4	4	4	4

TABLE 3--In Vitro and In Vivo Results of Rhesus Monkey RBC Frozen with 40% (w/v) Glycerol and Stored at -80 C, and Washed. The Washed RBC were Stored at 4 C for 24 Hours Prior to Autotransfusion

Monkey No.	Storage at -80 C (days)	Freeze-Thaw Recovery (%)	Freeze-Thaw-Wash Recovery (%)	24-Hour ^{51}Cr Survival (%)	ITE* (%)	RBC 2,3 DPG Day Wash (uM/g Hb)	RBC ATP Day Wash (uM/g Hb)	RBC K^+ Day Wash (mEq/10^{12} RBC)	RBC Na^+ Day Wash (mEq/10^{12} RBC)	Supt. Hb Day Wash (mg%)	Extra K^+ Day Wash (mEq/l)
1	42	98.6	91.1	80.0	72.9	15.1	3.3	6.93	2.37	39	0.3
2	42	99.1	ND+	83.0	ND	11.6	3.1	6.72	2.23	44	0.2
3	42	98.4	75.1	91.0	68.3	9.5	2.9	9.29	2.22	30	0.1
4	1	98.0	89.6	ND	ND	10.1	2.9	8.24	1.70	30	0.2
5	2	99.2	93.6	ND	ND	10.6	3.2	ND	ND	30	0.1
Mean		98.7	87.4	84.7	70.6	11.4	3.1	7.80	2.13	35	0.2
SD		0.5	8.3	5.7	3.2	2.2	0.2	1.20	0.29	7	0.1
SE		0.2	4.2	3.3	2.3	1.0	0.1	0.60	0.14	3	0.05
n		5	4	3	2	5	5	4	4	5	5

*Index of therapeutic effectiveness

+Not determined

FIGURE 1--The 24-hour and T_{50} values of ^{51}Cr-labeled monkey RBC collected in acid-citrate-dextrose (ACD, NIH, Formula A), labeled with disodium ^{51}Cr chromate, and reinfused within 4 hours of collection.

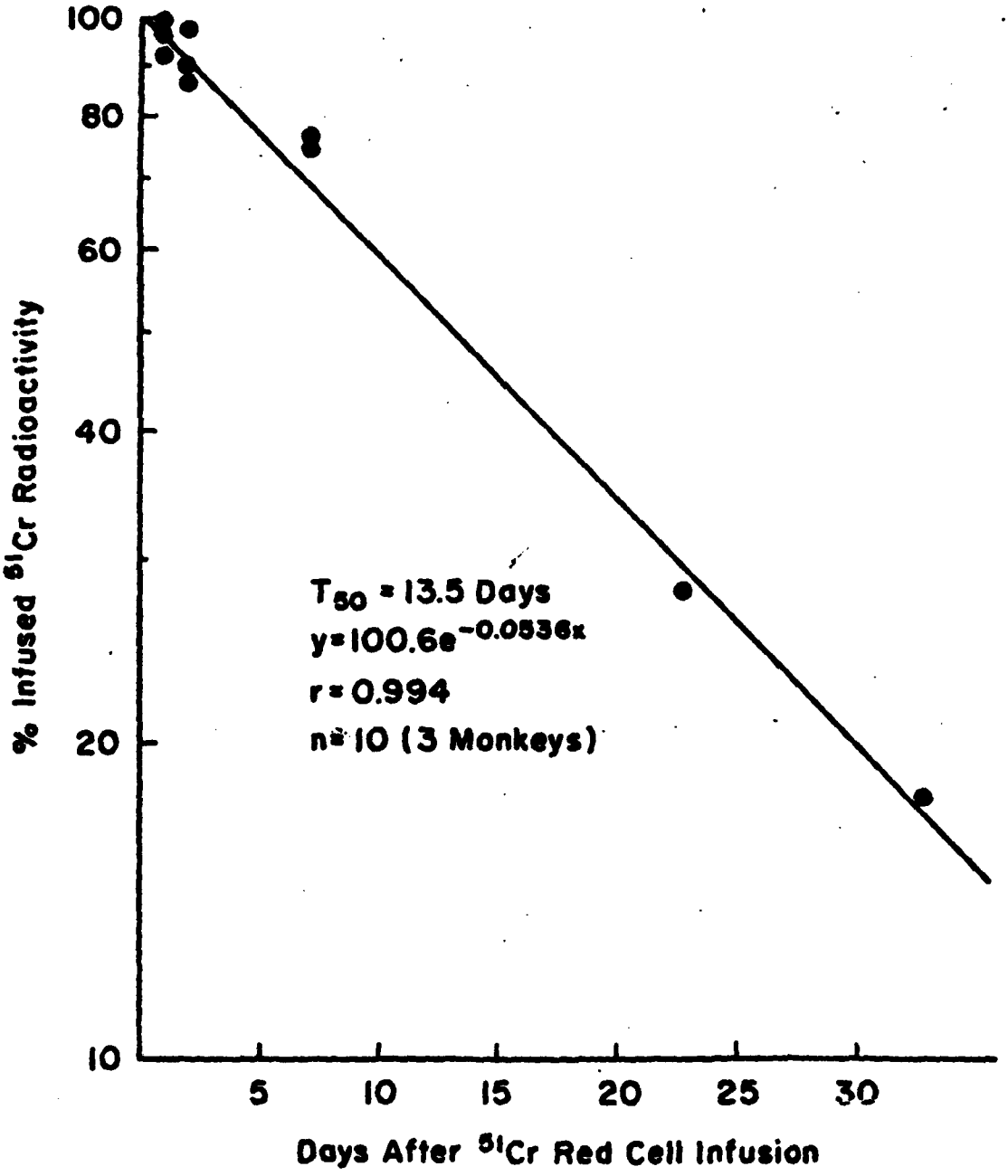

FIGURE 1

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FIGURE 2--The 24-hour and T_{50} values of monkey RBC frozen with 40% (w/v) glycerol and stored at -80 C, washed in the IBM Blood Processor 2991-1 or 2991-2 with sodium chloride solutions, and stored at 4 C in sodium chloride-glucose-phosphate for 24 hours prior to autotransfusion.

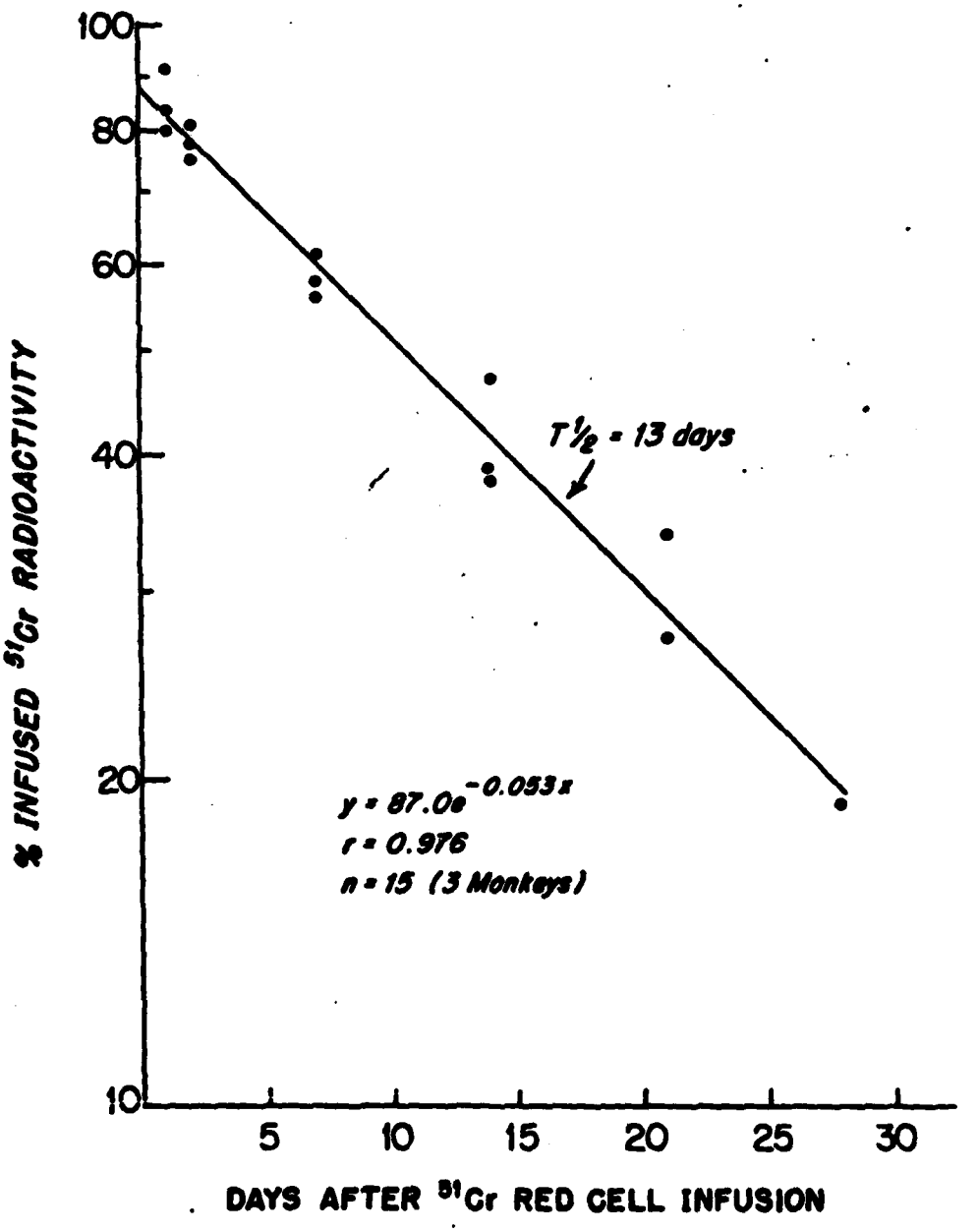

FIGURE 2

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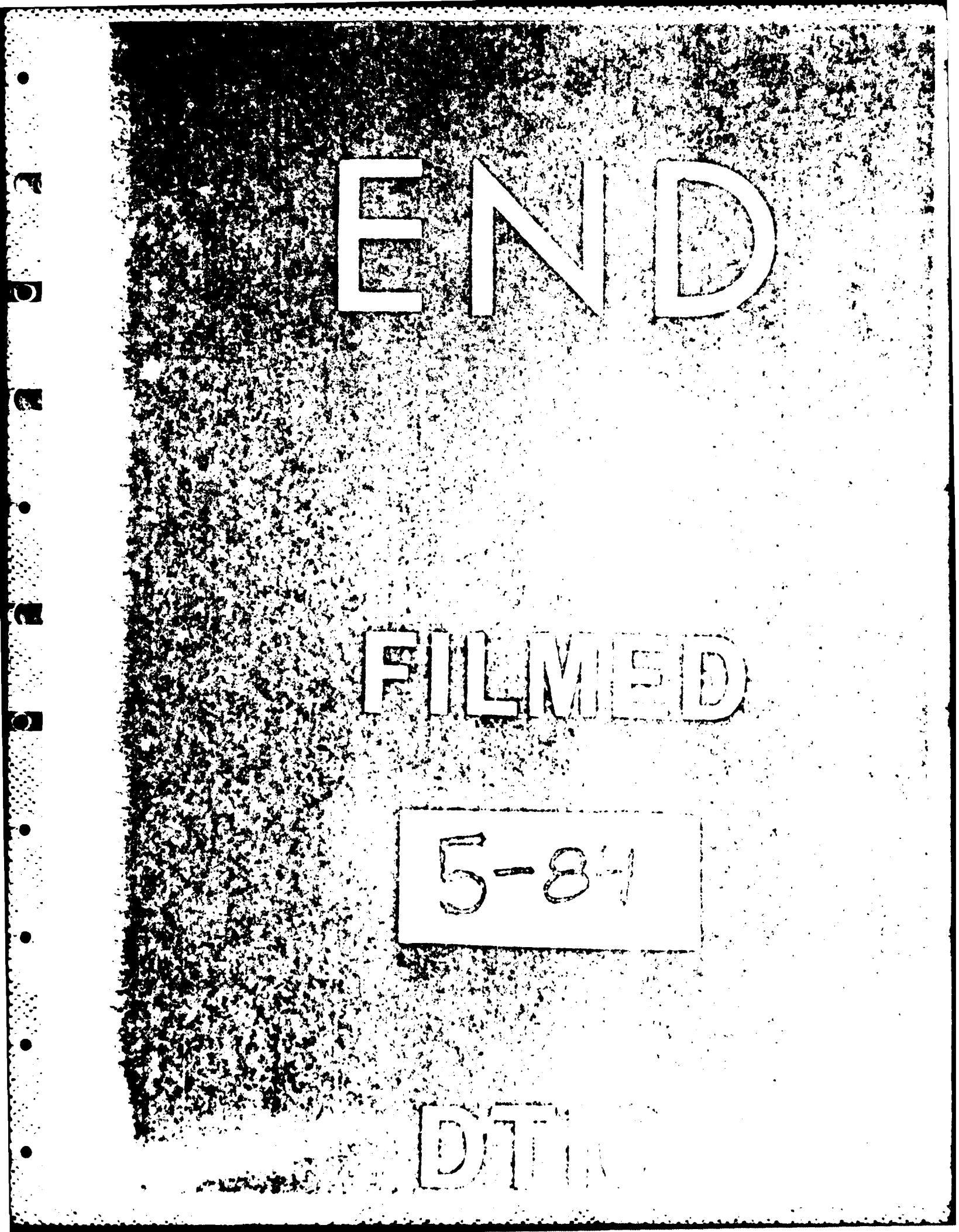
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